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Free Radical-induced Generation of Isoprostanes *in Vivo*

EVIDENCE FOR THE FORMATION OF D-RING AND E-RING ISOPROSTANES*

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Jason D. Morrow‡§¶, Tanya A. Minton‡, Chetan R. Mukundan‡, Michelle D. Campbell‡, William E. Zackert‡, Vincent C. Daniel‡, Kamal F. Badr‖, Ian A. Blair‡, and L. Jackson Roberts, II‡§**

From the Departments of ‡Pharmacology and §Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6602 and the ‖Division of Nephrology, Department of Medicine, Emory University and Veterans Affairs Medical Center, Atlanta, Georgia 30033

We recently reported the discovery that a series of novel prostaglandin (PG)F_2-like compounds (F_2-isoprostanes) are produced *in vivo* independent of the cyclooxygenase as products of free radical-catalyzed lipid peroxidation. F_2-isoprostanes are initially formed *in situ* from arachidonic acid esterified to phospholipids and then released preformed. We have now investigated whether PGD_2/E_2-like isoprostanes are also produced by rearrangement of the PGG_2-like intermediates involved in isoprostane formation. Using a variety of approaches utilizing mass spectrometry, compelling evidence was obtained for the presence of D_2/E_2-isoprostane-containing phosphospholipids in the liver (85 ± 33 ng/g of liver) and free D_2/E_2-isoprostanes in the circulation (215 ± 90 pg/ml) of rats treated with CCl_4 to induce lipid peroxidation. In untreated rats, levels of D_2/E_2-isoprostanes esterified in liver phospholipids were much lower (0.90 ± 0.10 ng/g), and free compounds could not be detected in the circulation (<5 pg/ml). Interestingly, one of the E_2-isoprostanes that would be expected to be formed in abundance, 8-epi-PGE_2, was found to be a potent renal vasoconstrictor, and these effects could be abrogated by SQ29548, a thromboxane receptor antagonist. Further understanding of the biological consequences of the formation of these novel compounds and factors that influence their formation may provide valuable insights into the pathophysiology of oxidant injury.

Free radical-catalyzed lipid peroxidation has been implicated in the pathogenesis of a wide variety of human disorders (1–4). Nonetheless, much remains to be understood about mechanisms of oxidant injury *in vivo*. Recently, we reported the discovery that a series of prostaglandin (PG)[1] F_2-like compounds (F_2-isoprostanes) capable of exerting potent biological activity are produced *in vivo* in humans as products of free radical-catalyzed peroxidation of arachidonic acid (5). Formation of these compounds occurs independently of the cyclooxygenase enzyme, which had heretofore been considered obligatory for endogenous prostanoid biosynthesis. Circulating levels of these compounds increase dramatically in animal models of free radical injury, and quantification of F_2-isoprostanes has proven to be an important advance in our ability to assess oxidant status *in vivo* (6). Formation of F_2-isoprostanes proceeds through intermediates comprised of four positional peroxyl radical isomers of arachidonic acid which undergo endocyclization to yield PGG_2-like bicyclic endoperoxides. The endoperoxides are then reduced to F-ring isoprostanes (5). Recently, we reported the finding that F_2-isoprostanes are primarily formed *in situ* from arachidonic acid esterified in phospholipids and subsequently released preformed, presumably by a phospholipase(s) (7). This mechanism of formation is in contradistinction to the formation of cyclooxygenase-derived prostanoids in which arachidonic acid esterified to phospholipids must first be released prior to oxygenation.

Because the formation of isoprostanes proceeds through endoperoxide intermediates, we investigated the possibility that, in addition to F-ring isoprostanes, compounds with a prostane D-ring and E-ring (D_2/E_2-isoprostanes) may also be formed *in vivo* by rearrangement of the endoperoxide intermediates. We present evidence that D_2/E_2-isoprostanes are, in fact, produced *in vivo*, that they are present both esterified to phospholipids and in free form, and that they are capable of exerting potent biological activity.

EXPERIMENTAL PROCEDURES

Reagents—Methoxyamine·HCl, pentafluorobenzyl bromide, diisopropylethylamine, and *Apis mellifera* venom phospholipase A_2 were obtained from Sigma. Dimethylformamide and undecane were obtained from Aldrich. *N,O*-Bis(trimethylsilyl)trifluoroacetamide was obtained from Supelco (Bellefonte, PA). [$^2H_9$]*N,O*-Bis(trimethylsilyl)trifluoroacetamide and [$^2H_3$]methoxyamine·HCl were obtained from Regis Chemical Co. (Morton Grove, IL). 1-Butaneboronic acid was obtained from Applied Science Laboratories, State College, PA. All organic solvents were obtained from Baxter Healthcare (Burdick and Jackson Brand, McGaw Park, IL). C-18 and silica Sep-Paks were purchased from Waters Associates (Milford, MA). TLC was performed on silica gel 60ALK6D plates (Whatman International Ltd., Maidstone, U. K.). The E_2-isoprostane 8-epi-PGE_2 (8) was a generous gift of Dr. Douglass Taber, University of Delaware (Newark, DE). [$^2H_7$]9α,11β-PGF_2 was biosynthesized as described previously (9). Other prostaglandin standards were obtained from Cayman Chemical (Ann Arbor, MI). Phosphatidylcholine with palmitate at the *sn*-1 position and [$^2H_7$]arachdonic acid at the *sn*-2 position was purchased from DuPont NEN.

Analysis of D2/E2-isoprostanes—D_2/E_2-isoprostanes were analyzed by gas chromatography (GC)/negative ion chemical ionization (NICI)-mass spectrometry (MS) using a modification of methods described previously for the analysis of PGD_2 and PGE_2 (10). Briefly, 1.5 ng of [$^2H_4$]PGE_2 internal standard is initially added to a biological fluid and adjusted to pH 3 with 1 M HCl. The sample is then applied to a C-18 Sep-Pak cartridge that has been prewashed with 5 ml of methanol and

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¶ Howard Hughes Medical Institute Physician Research Fellow and recipient of a career development award from the International Life Sciences Institute.

** To whom correspondence should be addressed: Dept. of Pharmacology, Vanderbilt University, Nashville, TN 37232-6602. Tel.: 615-322-3304; Fax: 615-322-4707.

[1] The abbreviations and trivial names used are: PG, prostaglandin; GC, gas chromatography; MS, mass spectrometry; NICI, negative ion chemical ionization; TMS, trimethylsilyl; PFB, pentafluorobenzyl; HPLC, high performance liquid chromatography; GFR, glomerular filtration rate; RPF, renal plasma flow.

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5 ml of H_2O (pH 3). The cartridge is then washed with 10 ml of H_2O (pH 3) followed by 10 ml of heptane, and compounds are then eluted with 10 ml of ethyl acetate and evaporated to dryness under nitrogen. Compounds are subsequently methoximated by treatment with 250 µl of a 2% solution of aqueous methoxyamine·HCl for 30 min at room temperature. Compounds are then extracted with 1 ml of ethyl acetate and the organic layer evaporated under nitrogen. Compounds are converted to a pentafluorobenzyl (PFB) ester by the addition of 40 µl of a 10% solution of pentafluorobenzyl bromide in acetonitrile and 20 µl of a solution of 10% diisopropylethanolamine in acetonitrile and allowed to incubate for 30 min at 37 °C. Reagents are dried under nitrogen and the residue reconstituted in 30 µl of chloroform and 20 µl of methanol and chromatographed on a silica TLC plate to 13 cm in a solvent system of ethyl acetate:methanol (98:2, v/v). The methyl ester of $PGF_{2\alpha}$ and the *O*-methyloxime, PFB ester derivative of PGD_2 (approximately 5 µg each) are chromatographed on a separate lane and visualized with 10% phosphomolybdic acid in ethanol by heating. The R_F of $PGF_{2\alpha}$ methyl ester in this solvent system is ~0.25, and the R_F of the *O*-methyloxime, PFB ester derivative of PGD_2 is ~0.60. Compounds migrating in the region 1 cm above the $PGF_{2\alpha}$ standard to 0.5 cm below the PGD_2 standard are scraped from the TLC plate, extracted with 1 ml of ethyl acetate, and dried under nitrogen.

Following TLC purification, compounds are converted to trimethylsilyl (TMS) ether derivatives by the addition of 20 µl of *N,O*-bis(trimethylsilyl)trifluoroacetamide and 10 µl of dimethylformamide. The sample is incubated at 37 °C for 10 min and then dried under nitrogen. The residue is redissolved for GC/MS analysis in 10 µl of undecane which has been stored over a bed of calcium hydride. GC/NICI-MS is carried out on a Nermag R10–10C mass spectrometer interfaced with a DEC-PDP computer. GC is performed using a 15-m, 0.25-µm film thickness, DB-1701 fused silica capillary column (J and W Scientific, Folsom CA). The column temperature is programmed from 190 to 300 °C at 20 °C/min. The major ion generated in the NICI mass spectra of the PFB ester, *O*-methyloxime, TMS ether derivative of PGD_2 and PGE_2, which would be the same ion generated by D_2/E_2-isoprostanes, is the *m/z* 524 carboxylate anion [M-181 (M-$^{\cdot}CH_2C_6F_5$)]. The corresponding ion generated by the [$^2H_4$]-PGE_2 internal standard is *m/z* 528. Levels of endogenous D_2/E_2-isoprostanes in a biological sample are calculated from the ratio of intensities of the ions *m/z* 524 to 528. In some experiments, compounds were reacted with 1-butaneboronic acid or subjected to catalytic hydrogenation following TLC purification as described (11). D_2/E_2-isoprostanes were also analyzed by GC/electron ionization-MS as methyl ester, *O*-methyloxime, TMS ether derivatives. Purification and derivatization of compounds for analysis by GC/electron ionization-MS was as noted above except the methyl ester derivatives were formed by treatment of compounds with excess ethereal diazomethane.

Analysis of F_2-isoprostanes—Purification, derivatization, and analysis of F_2-isoprostanes by GC/NICI-MS were performed as described (11).

Animal Model of Free Radical-induced Lipid Peroxidation—Free radical-catalyzed lipid peroxidation was induced in rats by intragastric administration of CCl_4 as described previously (12). In some cases, animals were pretreated with indomethacin with a dosing regimen shown previously to inhibit the cyclooxygenase >90% (5 mg subcutaneously at 24, 12, and 2 h prior to the administration of CCl_4) (13). At various time intervals, animals were sacrificed and the livers removed, snap frozen in liquid N_2, and either processed immediately or stored at −70 °C. In some experiments, blood was also removed from the animals and plasma processed and analyzed for isoprostanes as described above.

Extraction, Purification, and Hydrolysis of Phospholipids—Lipids from the livers of CCl_4-treated rats were extracted as described (14). Depending on the experiment, 0.005% butylated hydroxytoluene was added to the lipid extracts during the extraction procedure. The lipid extracts (containing approximately 1 µmol of phospholipid) were then hydrolyzed by reaction with *A. mellifera* venom phospholipase A_2 (approximately 200 µg) as described (15) and subsequently analyzed for free D_2/E_2-isoprostanes. As a positive control for phospholipase A_2 activity, phosphatidylcholine containing [^{3}H]arachidonate in the *sn*-2 position was added to the incubation mixture, and the percent of radiolabeled arachidonate released was determined as described (7). In all experiments > 95% of esterified [^{3}H]arachidonate was released. In some experiments, phospholipid extracts were first reduced with $NaBH_4$ and then hydrolyzed using methanolic KOH (7, 11).

Analysis of Lipid Extracts from Livers of CCl_4-treated Rats by Normal Phase HPLC—Normal phase HPLC analysis of lipid extracts was performed on a 25-cm × 4.6-mm Econosil SI column (Alltech, Deerfield IL) with 5-µm particles using an isocratic solvent system of hexane: isopropyl alcohol:water (4:6:1, v/v/v) at a flow rate of 1 ml/min. UV absorbance was monitored continuously at 205 nm. Aliquots of fractions eluted were subjected to hydrolysis using bee venom phospholipase A_2 and then analyzed for free D_2/E_2-isoprostanes as described above.

Analysis of Phospholipids by Negative Liquid Secondary Ion Tandem Mass Spectrometry—Negative liquid secondary ion MS and tandem MS (MS/MS) were carried out on a Kratos Concept II HH four-sector tandem mass spectrometer. The instrument was operated with an accelerating voltage of 8 kV. It was equipped with a cesium ion gun that was operated at 5 kV. Samples were dissolved in a matrix of 20% triethanolamine in dimethyl sulfoxide. Collision-induced dissociation was performed in the collision cell situated at the focal point between the two mass spectrometers. Argon was used as the collision gas at a pressure of 5×10^{-5} torr with the collision cell floated at 4 kV. Precursor and product ion spectra were obtained using the Sun Mach III data system; four to six scans were averaged for each spectrum.

Biological Effects of 8-Epi-PGE_2 in the Kidney—Renal clearance studies were performed using 200–250 male Sprague-Dawley rats as described elsewhere (16). In the first group, time control rats (n = 3), base-line measurements were performed and repeated during a 30-min infusion of vehicle (0.1 N phosphate-buffered saline). In the second group of rats (n = 9), whole kidney measurements were performed during base-line conditions and during the infusion of 8-epi-PGE_2 in the following doses; 1.0 µg/kg/min (n = 3), 2.0 µg/kg (n = 3), and 4.0 µg/kg (n = 3). In a third group of rats (n = 6), 8-epi-PGE_2 was administered at a rate of 2.0 µg/kg/min (n = 3) or 4.0 µg/kg/min (n = 3) in the presence of a thromboxane A_2 receptor antagonist, SQ29548, administered by a continuous intravenous infusion of 3 mg/kg/h. Treatment with SQ29548 was started 30 min before the initial base-line measurements. Glomerular filtration rate (GFR), renal plasma flow (RPF), and arterial blood pressure values were expressed as mean ± S.E. of three determinations. Intergroup multiple comparisons were performed with one-way analysis of variances followed by Dunnett's multiple comparison procedure, while, for nonmultiple comparison, Student's unpaired t test was done. Differences were considered significant at $p < 0.05$.

RESULTS

Evidence for the Formation of D_2/E_2-isoprostanes in Vitro during Storage of Plasma—Previously, we had shown that plasma arachidonic acid undergoes autoxidation during storage of plasma at −20 °C for several months, resulting in the formation of large quantities of F_2-isoprostanes (11). Concentrations of F_2-isoprostanes in fresh plasma that has not been stored are approximately 20 pg/ml, and storage of plasma for several months increases levels consistently to greater than 1,000 pg/ml. Therefore, we initially explored whether D_2/E_2-isoprostanes are also formed *in vitro* by analyzing plasma that had been subjected to storage at −20 °C for approximately 6 months. The selected ion current chromatograms obtained from this analysis monitoring *m/z* 524 for D_2/E_2-isoprostanes and *m/z* 528 for the [$^2H_4$]PGE_2 internal standard are shown in Fig. 1. When analyzed in an analogous fashion, F_2-isoprostanes are detected as a series of peaks eluting from the GC column over approximately a 30-s interval (11). Similarly, in the *upper m/z* 524 chromatogram in Fig. 1, a series of peaks are present which eluted from the GC column over an approximate 40-s interval. The two peaks in the *lower m/z* 528 chromatogram represent *syn* and *anti* *O*-methyloxime isomers of [$^2H_4$]PGE_2. Quantification of compounds in the *m/z* 524 chromatogram is based on a comparison of the ratios of the *starred* (★) *m/z* 524 peak with that of the *starred* (★) peak in the *m/z* 528 chromatogram. The concentration of the compounds in the *m/z* 524 chromatogram was calculated to be 2,500 pg/ml. Four additional plasma samples that had been stored for various periods of time were then analyzed, and the concentrations found ranged from between 90 and 21,000 pg/ml. In contrast, these compounds could not be detected (lower limits of detection = 5 pg/ml) when fresh plasma from normal volunteers was analyzed, suggesting that the compounds detected in stored plasma were formed during storage.

The finding that large quantities of a series of compounds were formed during storage of plasma that had TLC and GC/MS properties similar to those of PGE_2 would be consistent

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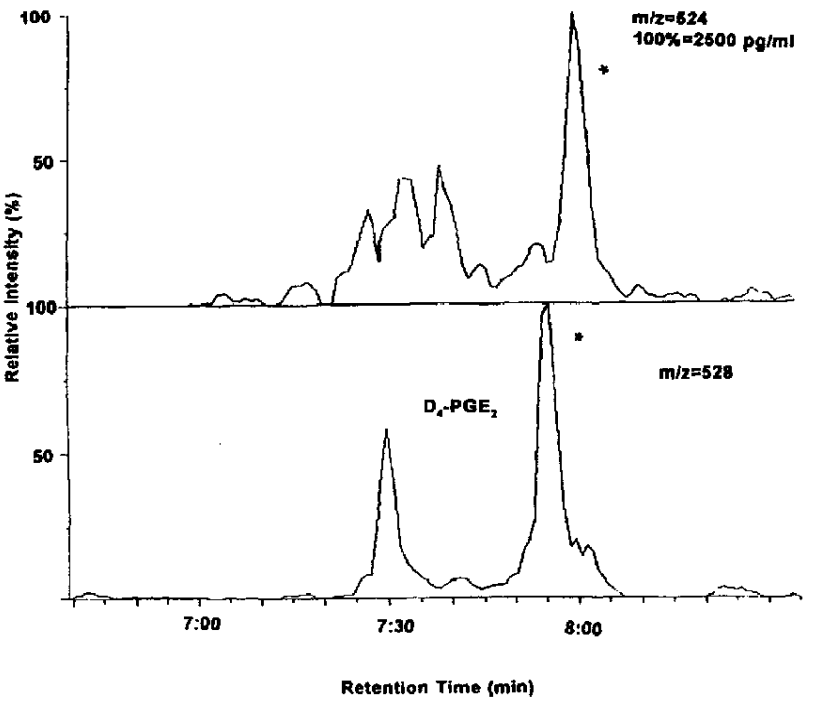

FIG. 1. **Analysis of plasma which had been stored at −20 °C for several months for D_2/E_2-isoprostanes by GC/NICI-MS.** The two peaks in the *m/z* 528 ion current chromatogram represent *syn* and *anti* *O*-methyloxime isomers of the [$^2H_4$]PGE$_2$ internal standard. In the *m/z* 524 ion current chromatogram are a series of peaks consistent with the presence of D_2/E_2-isoprostanes. Levels of compounds are based on a comparison of the ratio of the *starred* (★) peak in the *m/z* 524 chromatogram to the *starred* (★) peak in the *m/z* 528 chromatogram.

with their being D/E-ring isoprostanes. Since PGD$_2$ and PGE$_2$ have similar chromatographic properties on TLC and GC and have identical molecular weights and thus generate the same major M-181 ion when analyzed by NICI-MS, it is not possible to differentiate whether the putative isoprostane compounds detected in the *m/z* 524 ion current chromatogram in Fig. 1 have a D-type or E-type prostane ring. However, since the endoperoxide PGH$_2$ derived from the cyclooxygenase rearranges in aqueous solutions to form both PGD$_2$ and PGE$_2$ (17), one would expect that the isoprostane endoperoxide intermediates would also rearrange to form both D-ring and E-ring isoprostanes.

Additional experimental approaches were then employed in an attempt to obtain further evidence that the compounds detected in stored plasma were D_2/E_2-isoprostanes. First, no peaks were present when *m/z* 523 was monitored, indicating that the *m/z* 524 peaks were not natural isotope peaks of compounds generating an ion less than 524 Da. When the compounds were analyzed as [$^2H_9$]TMS ether derivatives, the *m/z* 524 peaks all shifted upwards 18 Da, indicating the compounds have 2 hydroxyl groups. When the compounds were analyzed as a [$^2H_3$] *O*-methyloxime derivative, the *m/z* 524 peaks all shifted upward 3 Da, indicating that they contain one carbonyl group. These data thus indicate that these compounds have the same functional groups as PGD$_2$ and PGE$_2$ and are consistent with their being D_2/E_2-isoprostanes.

Analysis for the Presence of D_2/E_2-isoprostanes Esterified to Phospholipids in Vivo—Since the above results suggested that D_2/E_2-isoprostanes could be formed *in vitro*, we investigated whether these compounds may also be formed *in vivo*. Previously, we had shown that F_2-isoprostanes are initially formed *in situ* from arachidonic acid esterified in tissue phospholipids and subsequently released preformed (7). Therefore, we examined whether D_2/E_2-isoprostanes might be esterified to phospholipids in livers of rats that had been treated with CCl_4 to induce lipid peroxidation. To investigate this, lipids were extracted from the livers, subjected to hydrolysis using *A. mellifera* venom phospholipase A$_2$, and subsequently analyzed for free compounds. Enzymatic hydrolysis of phospholipids was employed to circumvent the problem of dehydration of the prostane D/E-rings during chemical hydrolysis with strong base. The results of this analysis are shown in Fig. 2. A series of *m/z* 524 peaks were present in a pattern very similar to that obtained from analysis of stored plasma, although the relative abundances of the various peaks differ somewhat (*cf.* Fig. 1). Table I compares the amounts of the putative D_2/E_2-isoprostanes with the amounts of F_2-isoprostanes measured following hydrolysis of lipids from the same livers of both untreated and CCl_4-treated rats. The quantities of D_2/E_2-isoprostanes measured following hydrolysis of lipids from livers of CCl_4-treated rats were approximately 100-fold higher than those in untreated rats. Levels of free D_2/E_2-isoprostanes measured in lipid extracts that were not subjected to hydrolysis were < 1% of the levels measured following hydrolysis ($n = 4$), suggesting that the compounds detected following treatment with phospholipase A$_2$ were released from an acyl linkage on phospholipids. Pretreatment of animals with indomethacin prior to CCl_4 administration with a dosage regimen shown previously to inhibit cyclooxygenase activity by >90% (13) did not affect levels of the compounds measured ($p > 0.8$, Student's t test, $n = 4$), indicating that the cyclooxygenase enzyme is not involved in their formation. Previously we had shown that butylated hydroxytoluene markedly suppresses the formation of F_2-isoprostanes by autoxidation *in vitro* (11). The presence of butylated hydroxytoluene (0.005%) in the extraction solution, however, did not affect levels of D_2/E_2-isoprostanes measured ($p > 0.8$, $n = 4$), arguing that these compounds are not formed *ex vivo* by autoxidation during sample processing.

Experiments were then carried out to obtain further evidence for the identity of the compounds represented by the *m/z* 524 peaks in Fig. 2 as D_2/E_2-isoprostanes. First, analysis of compounds obtained from the hydrolysis of liver extracts as a [$^2H_9$]TMS ether derivative resulted in a shift of each of the *m/z* 524 peaks upward 18 Da, indicating the presence of two hy-

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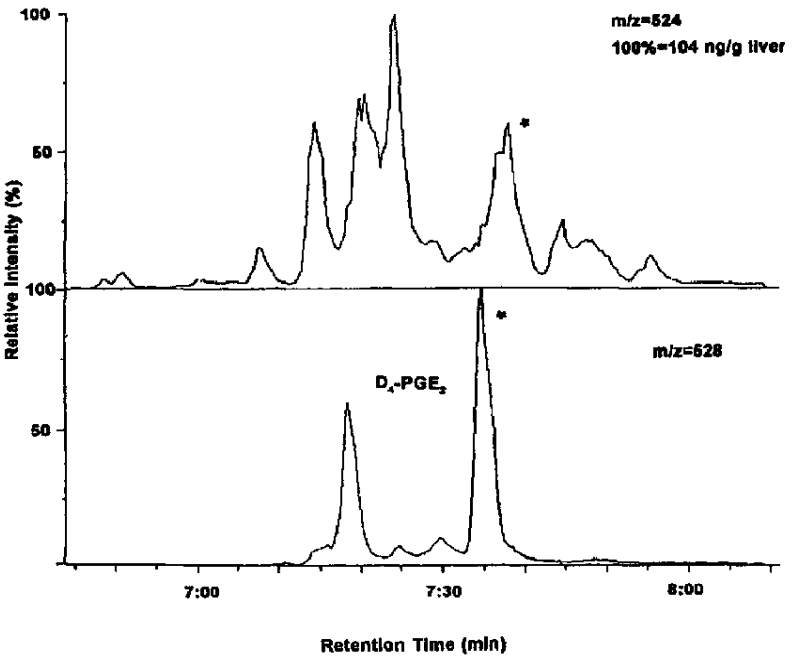

FIG. 2. **Analysis for D_2/E_2-isoprostanes by GC/NICI-MS following hydrolysis of a lipid extract from the liver of a rat treated with CCl_4 to induce lipid peroxidation.** A series of peaks are present in the *m/z* 524 ion current chromatogram representing putative D_2/E_2-isoprostanes; the *m/z* 528 chromatogram represents the $[^2H_4]PGE_2$ internal standard. Levels of compounds are based on a comparison of the ratio of the *starred* (★) peak in the *m/z* 524 chromatogram to that in the *m/z* 528 chromatogram.

TABLE I
Levels of isoprostanes in hydrolyzed lipid extracts from liver tissue of rats with and without CCl_4 treatment (n = 6 animals/group)

	D_2/E_2-isoprostanes	F_2-isoprostanes
	ng/g liver	
Untreated rats	0.90 ± 0.10	2.8 ± 0.4
CCl_4-treated rats	85 ± 33	459 ± 126

droxyl groups. Analysis as a $[^2H_3]O$-methyloxime derivative resulted in a shift of the *m/z* 524 peaks upward 3 Da, indicating the presence of one carbonyl group. When the compounds were analyzed following catalytic hydrogenation, there was a disappearance of the *m/z* 524 peaks and the appearance of new intense peaks 4 Da higher at *m/z* 528 (Fig. 3). No peaks were detected at *m/z* 526 or 530. This indicated that all of the compounds contained two double bonds. Collectively, these results indicated that the compounds represented by the *m/z* 524 peaks contain the same functional groups and number of double bonds as would be expected for D/E-ring isoprostanes.

An additional experimental approach was employed to obtain further evidence for the presence of D/E-ring isoprostanes esterified to phospholipids. In this experiment, phospholipids extracted from livers of CCl_4-treated rats were first reduced by treatment with $NaBH_4$ and then subjected to hydrolysis with methanolic KOH. The hydrolysate was then analyzed for the presence of F_2-isoprostanes with *trans*-cyclopentane ring hydroxyls. The rationale of this experimental approach is based on the fact that we had shown previously that the cyclopentane ring hydroxyls of F_2-isoprostanes that are produced *in vivo* are exclusively oriented *cis* (5, 7). Chemical reduction of the carbonyl on D/E-ring compounds, however, would result in a mixture of F-ring compounds with *cis*- and *trans*-cyclopentane ring hydroxyls. Thus, a finding of F_2-isoprostanes with cyclopentane ring hydroxyls oriented *trans* in the hydrolysate of phospholipids that had first been reduced with $NaBH_4$ would provide compelling indirect evidence that D/E-ring isoprostanes were present esterified to the phospholipids. Orientation of the hydroxyls on F-ring prostanoids can be assessed by determining whether a cyclic boronate derivative bridging the ring hydroxyls forms when treated with 1-butaneboronic acid. A cyclic boronate derivative will form only if the cyclopentane ring hydroxyls are oriented *cis* (18).

The results of the analyses for F_2-isoprostanes hydrolyzed from phospholipids that had been reduced with $NaBH_4$ are shown in Fig. 4. At the *top* of Fig. 4*A* is shown the *m/z* 569 selected ion current chromatogram obtained from the analysis for F_2-isoprostanes hydrolyzed from phospholipids that had not been reduced with $NaBH_4$. In this analysis, compounds were not treated with 1-butaneboronic acid. In the *lower three* chromatograms are the results obtained following treatment of these compounds with *n*-butylboronic acid. The major ion generated by F_2-isoprostanes as a PFB ester, TMS ether, cyclic butylboronate derivative is the M-181 ion, *m/z* 491 (11). As is evident, the *m/z* 569 peaks disappear when reacted with 1-butaneboronic acid, and there is a coincident appearance of intense peaks in the *m/z* 491 chromatogram shown at the *bottom* of Fig. 4*A*. These results thus indicate that essentially all of the F_2-isoprostanes formed a cyclic boronate derivative. Fig. 4*B* shows the results of similar analyses for F_2-isoprostanes hydrolyzed from phospholipids that had first been reduced with $NaBH_4$. The *m/z* 569 chromatogram at the *top* of Fig. 4*B* was obtained when the compounds were not treated with 1-butaneboronic acid. The pattern of the *m/z* 569 peaks is similar to that obtained from the analysis of the hydrolysate of lipids that had not been reduced (Fig. 4*A*, *top* chromatogram). However, the level of compounds measured is approximately 1.6-fold higher in 4*B* than in 4*A*. In the *lower* set of three chromatograms are the results obtained from the analysis of these compounds following treatment with 1-butaneboronic acid. Similar to the results in Fig. 4*A*, intense *m/z* 491 peaks appear (Fig. 4*B*, *bottom* chromatogram), indicative of compounds that formed a cyclic boronate derivative. In contrast to the results shown in Fig. 4*A*, however, peaks also remain in the *m/z* 569 chromatogram after treatment with 1-butaneboronic acid. This suggested the presence of F-ring compounds with *trans* cyclopentane hydroxyls. Of note, the quantity of the compounds that did

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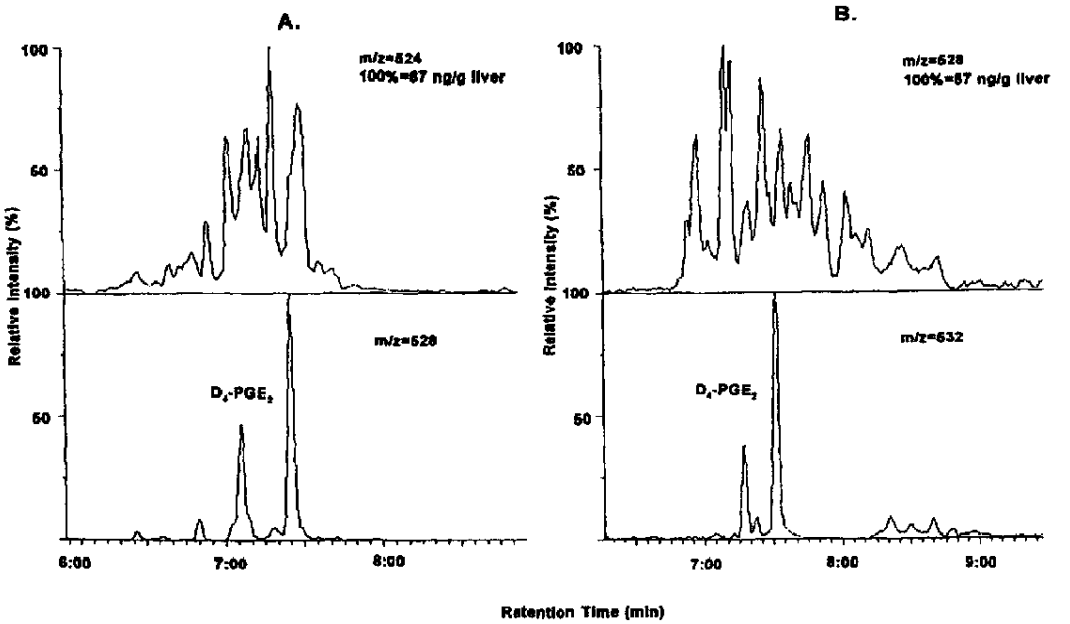

FIG. 3. **Catalytic hydrogenation of putative D_2/E_2-isoprostanes in the hydrolysate of a lipid extract from the liver of a rat treated with CCl_4.** *Panel A*, GC/NICI-MS analysis of compounds that have not been subjected to catalytic hydrogenation. Peaks in the *m/z* 524 chromatogram represent the putative D_2/E_2-isoprostanes, and the peaks in the *m/z* 528 chromatogram represent the [$^2H_4$] PGE_2 internal standard. *Panel B*, analysis of compounds following hydrogenation. Both the internal standard and the *m/z* 524 peaks in *panel A* have shifted upward 4 Da following hydrogenation, indicating the presence of two double bonds. The pattern of *m/z* 524 peaks in *panel B* differs somewhat from the pattern of *m/z* 524 peaks in *panel A*. This is likely attributed to different GC characteristics of the compounds in which the double bonds have been reduced.

not form a cyclic boronate derivative was calculated to be approximately 215 ng/g of liver. Since chemical reduction of the carbonyl on D/E-ring compounds produces a mixture of compounds with both *cis*- and *trans*-cyclopentane hydroxyls, it can be estimated that the total quantity D/E-ring compounds that were present would be approximately 215 × 2 = 450 ng/g of liver. This figure is almost exactly the difference found in the levels of F-ring compounds in hydrolysates of lipids that had been reduced with $NaBH_4$ (1230 ng/g of liver; Fig. 4*B*, *top* chromatogram) and the levels measured in the hydrolysate of lipids that had not been reduced by $NaBH_4$ (750 ng/g of liver, Fig. 4*A*, *top* chromatogram). This suggests that the quantity of D/E-ring isoprostanes that are formed esterified to phospholipids *in vivo* is approximately 40% of the quantity of esterified F_2-isoprostanes that are formed. However, this may be somewhat of an underestimate. For our analyses of D/E-ring isoprostanes, we have arbitrarily scraped the region of the TLC plate which lies between the location where the methyl ester of $PGF_{2\alpha}$ migrates up to 0.5 cm below the location where the *O*-methyloxime, PFB ester derivative of PGD_2 migrates. However, we have also analyzed the region on the TLC plate 0.5 cm below to 1 cm above where the *O*-methyloxime, PFB ester of PGD_2 migrates for D_2/E_2-isoprostanes and have detected additional *m/z* 524 peaks that also elute from the GC with a retention time similar to that of PGE_2. Although these compounds may likely represent additional D/E-ring isoprostanes, they have not been scrutinized carefully, *e.g.* as to the number and type of functional groups that they contain, etc. Therefore, we cannot conclude with confidence that these compounds represent additional D_2/E_2-isoprostanes. We have noted, however, that the levels of these compounds increase in parallel in animal models of oxidant injury with the *m/z* 524 peaks routinely quantified.

Analysis for the Presence of Free Concentrations of D_2/E_2-isoprostanes in the Circulation in CCl_4-treated Rats—We have demonstrated previously that F_2-isoprostanes are initially formed esterifed to tissue phospholipids in CCl_4-treated rats and subsequently released into the circulation preformed (7). Since the above results suggested that D_2/E_2-isoprostanes are also formed esterified to phospholipids, we examined whether increased concentrations of these isoprostanes could also be detected free in the circulation of rats 4 h following administration of CCl_4 to induce lipid peroxidation. As was found in fresh human plasma, compounds presumably representing D/E-ring isoprostanes could not be detected in plasma from normal rats that had not been treated with CCl_4 (lower limit of detection = 5 pg/ml, $n = 4$). However, following treatment of rats with CCl_4, putative D_2/E_2-isoprostanes were detected in plasma at concentrations of 215 ± 90 pg/ml ($n = 4$), representing an increase of greater than 43-fold. The pattern of peaks was essentially identical to that shown in Fig. 2. Concentrations of F_2-isoprostanes measured in the same plasma samples were 640 ± 70 pg/ml ($n = 4$). The increased levels of D/E-ring isoprostanes in the circulation of CCl_4-treated rats were not affected by pretreatment of animals with indomethacin (266 ± 72 pg/ml ($n = 3$), $p > 0.5$, Student's *t* test). As before, analysis of these compounds in the circulation as deuterated TMS ether and deuterated *O*-methyloxime derivatives also confirmed that they had, as expected, two hydroxyl groups and one carbonyl group.

Although the above results were consistent with the identity of the compounds detected in the circulation as D_2/E_2-isoprostanes, we also considered the possibility that they might represent metabolites of F_2-isoprostanes in which the side chain hydroxyl had been metabolized by a dehydrogenase to a keto group. First, analogous to cyclooxygenase-derived $PGF_{2\alpha}$ in which metabolism to 15-keto compounds represents a major pathway of metabolism (19), a dehydrogenase may also be involved in metabolism of F_2-isoprostanes. In fact, we reported recently the identification of urinary metabolites of F_2-isopros-

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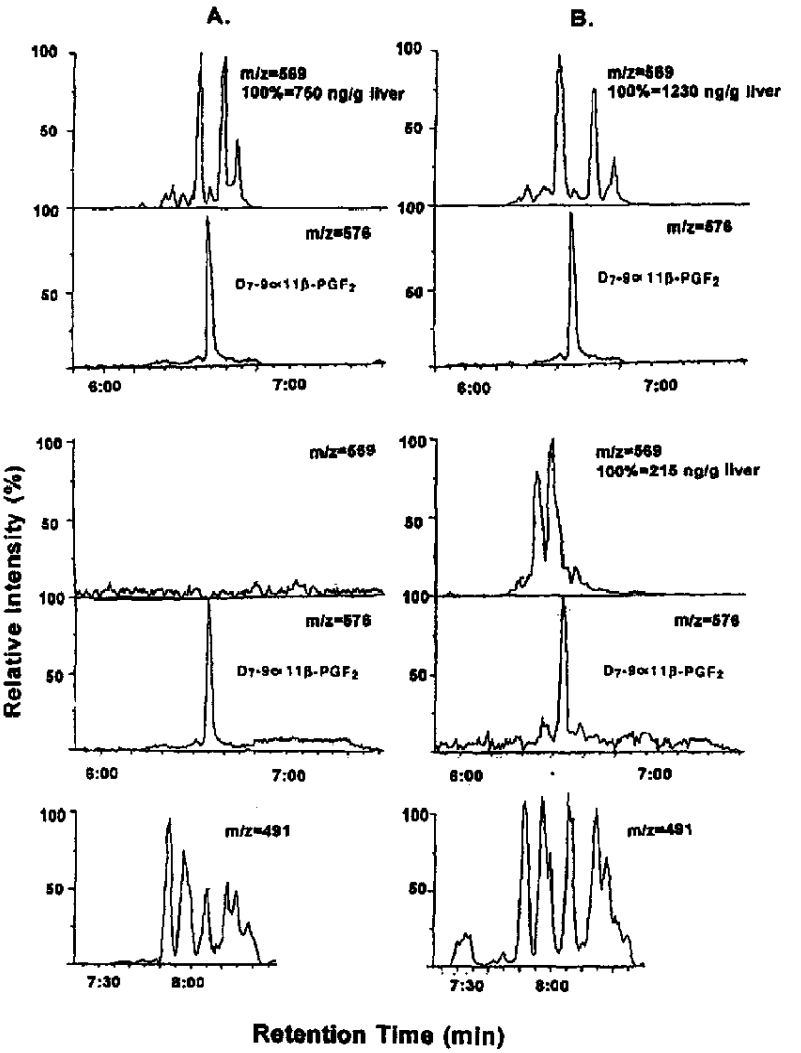

FIG. 4. **GC/NICI-MS analysis of F-ring isoprostanes hydrolyzed by methanolic KOH from a lipid extract obtained from the liver of a rat treated with CCl_4.** Compounds were analyzed both with and without treatment with 1-butaneboronic acid to assess whether compounds would form a cyclic boronate derivative. Peaks in the *m/z* 569 ion current chromatograms represent F-ring isoprostanes that either were not treated with 1-butaneboronic acid or did not form a cyclic boronate derivative. The peak in the *m/z* 576 ion current chromatograms represents the $[^2H_7]9\alpha,11\beta$-PGF_2 internal standard. Peaks present in the *m/z* 491 ion current chromatograms represent F-ring isoprostanes that formed a cyclic boronate derivative. *Panel A*, analysis of a hydrolysate of liver lipids that had not been reduced with $NaBH_4$ prior to hydrolysis. The *top two* ion current chromatograms represent F_2-isoprostanes that had not been treated with 1-butaneboronic acid. The *bottom three* ion current chromatograms are the results obtained when compounds in the hydrolysate were treated with 1-butaneboronic acid. *Panel B*, analysis of a hydrolysate of liver lipids that had been reduced with $NaBH_4$ prior to hydrolysis. The *top two* ion current chromatograms represent F_2-isoprostanes that had not been treated with 1-butaneboronic acid. The *bottom three* ion current chromatograms are the results obtained when compounds in the hydrolysate were treated with 1-butaneboronic acid.

tanes which contained a keto group (20). Second, metabolites of F_2-isoprostanes with a keto group would have the same functional groups as D_2/E_2-isoprostanes, *i.e.* two hydroxyl groups and one keto group. These metabolites, therefore, would generate the same M-181 ion, *m/z* 524, as D_2/E_2-isoprostanes when analyzed as a PFB ester, *O*-methyloxime, TMS ether derivative by GC/NICI-MS. However, it should be possible to differentiate metabolites of F-ring isoprostanes with a keto group on a side chain from D_2/E_2-isoprostanes by determining whether the compounds form a cyclic boronate derivative. Cyclic boronate derivatives will only form bridging vicinal hydroxyls or hydroxyls separated by no more than a single carbon atom. Thus, F_2-isoprostane metabolites will form a cyclic boronate derivative, whereas D_2/E_2-isoprostanes will not. When we treated the compounds detected in the circulation with 1-butaneboronic acid, they did not form a cyclic boronate derivative (not shown). This result suggested that these compounds were not keto metabolites of F_2-isoprostanes but were D/E-ring isoprostanes.

Analysis of Lipid Extracts by HPLC for Phospholipids Containing D_2/E_2-isoprostanes—The results obtained up to this point provided evidence suggesting strongly that D_2/E_2-isoprostanes are formed *in vivo* esterified to phospholipids. We had demonstrated previously that F_2-isoprostane-containing phospholipids exhibited much more polar characteristics on normal phase HPLC than nonoxidized phospholipids (7). One would expect, therefore, that D/E-ring isoprostane containing phospholipids would also exhibit polar characteristics on HPLC. Therefore, to substantiate further the existence of D/E-ring isoprostane-containing phospholipids, lipid extracts from livers of CCl_4-treated rats were subjected to normal phase HPLC analysis using a a solvent system that separates phosphatidylcholine from other phospholipids and neutral lipids (21–23). To detect lipids containing esterified D_2/E_2-isoprostanes, aliquots of fractions collected were hydrolyzed with bee venom phospholipase A_2 and free D_2/E_2-isoprostanes quantified by GC/MS. Consistent with the presence of D/E-ring isoprostane-containing species of phosphatidylcholine, fractions in which the majority (~70%) of free D_2/E_2-isoprostanes were detected after hydrolysis eluted at a much more polar retention volume (25–41 ml) than nonoxidized phosphatidylcholine, which eluted between 14 and 22 ml (Fig. 5). In addition, fractions also eluted between 2 and 8 ml in which free D_2/E_2-isoprostanes were detected after hydrolysis. Although not analyzed further, these were thought likely to represent phospholipid species other

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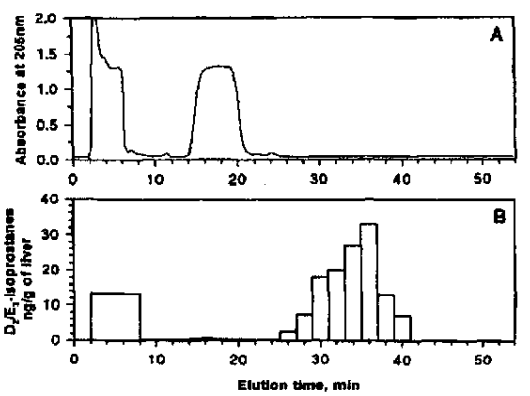

FIG. 5. **Normal phase HPLC analysis of lipid extracts from livers of CCl_4-treated rats.** UV absorbance was monitored continuously at 205 nm. The major UV peak eluted between 14 and 22 ml in chromatogram *A* represents unoxidized phosphatidylcholine, whereas the peaks eluted near the solvent front between 2 and 8 ml represent less polar phospholipids. In chromatogram *B* is shown the quantities of free D_2/E_2-isoprostanes detected by GC/MS after bee venom hydrolysis of aliquots of fractions that were collected and pooled as indicated by the widths of the *bars*. As noted, the majority of D_2/E_2-isoprostane containing lipids eluted at a more polar retention time (25–41 ml) than unoxidized phosphatidylcholine.

than phosphatidylcholine-containing esterified D_2/E_2 isoprostanes.

Analysis of D/E-ring Isoprostanes by Electron Ionization-MS—To obtain more direct evidence that the compounds analyzed by selected ion monitoring MS were D_2/E_2-isoprostanes, the compounds hydrolyzed from the polar lipids eluted from the HPLC column shown in Fig. 5 (from about 20 g of liver tissue) were analyzed as a methyl ester, *O*-methyloxime, TMS ether derivative by electron ionization MS. This analysis yielded multiple similar mass spectra of compounds eluting over approximately a 40-s period from the capillary GC column with characteristics of the electron ionization mass spectra of the same derivative of PGD_2 and PGE_2 (24). A mass spectrum obtained from the major eluting peak is shown in Fig. 6. The other mass spectra obtained were similar to that shown in Fig. 6, differing primarily in the relative ion abundances of some of the fragment ions. In the mass spectrum shown, there is an intense molecular ion present at the expected *m/z* 539. Other characteristic high mass ions present are: *m/z* 524 (M-15, loss of $^{\cdot}CH_3$); *m/z* 508 (M-31, loss of $^{\cdot}OCH_3$); *m/z* 468 (M-71, loss of $^{\cdot}CH_2(CH_2)_3CH_3$); *m/z* 449 (M-90, loss of Me_3SiOH); *m/z* 438 (M-101, loss of $^{\cdot}CH_2(CH_2)_2COOCH_3$), *m/z* 418 [M-(90+31)], *m/z* 398 (M-141, loss of $^{\cdot}CH_2CH{=}CH(CH_2)_3COOCH_3$), and *m/z* 378 [M-(90+71)]. The intense ions resulting from the loss of 71 Da and the ion at *m/z* 398 resulting from the loss of 141 Da suggests that this compound has the same basic structure as cyclooxygenase-derived prostaglandins with a cyclopentane ring at carbons C-8 through C-12. Prominent low mass ions are also present which are also prominent ions in the mass spectra of PGE_2 and PGD_2 such as *m/z* 199 representing the lower side chain ($^{+}CH{=}CH\text{-}CH(Me_2SiOH)(CH_2)_4CH_3$) and *m/z* 173 ($Me_3SiO^{+}{=}CH(CH_2)_4CH_3$). The origins of the other low mass ions present remain to be established but presumably arise from coeluting different positional isomeric compounds that are present in this mixture. These data thus provided further evidence for the identity of these compounds as D_2/E_2-isoprostanes.

Analysis of D_2/E_2-Isoprostane-containing Phospholipids by Negative Liquid Secondary Ion Tandem Mass Spectrometry—We sought to obtain further direct evidence for the presence of phospholipids containing esterified D_2/E_2-isoprostanes by analyzing the polar lipids eluted from the HPLC column (see Fig. 5) by negative liquid secondary ion mass spectrometry. The negative liquid secondary ion mass spectrum of the these phospholipid species showed expected high mass intense ions at *m/z* 843 and 798, corresponding to M-15 $[M\text{-}CH_3]^-$ and M-60 $[M\text{-}HN(CH_3)_3]^-$, respectively, of phosphatidylcholine containing stearate and a D_2/E_2-isoprostane (7, 25, 26). An expected ion at *m/z* 772 $[M\text{-}CH_2CHN(CH_3)_3]^-$ was not sufficiently intense to be observed above background chemical noise. Other structurally significant fragment ions were also present including an ion at *m/z* 351, corresponding to the carboxylate anion of a D_2/E_2-isoprostane, and an ion at *m/z* 283, corresponding to the carboxylate anion of stearic acid. Collision-induced dissociation on *m/z* 843 resulted in the formation of a series of structurally relevant daughter ions (Fig. 7). In particular, the ions at *m/z* 283 and 351 confirmed the presence of stearic acid and a D_2/E_2-isoprostane, respectively. The prominent ion at *m/z* 208 represents the phosphatidylcholine backbone resulting from the loss of the D_2/E_2-isoprostane and stearate. Collision-induced dissociation on *m/z* 351 produced daughter ions at *m/z* 333 ($M\text{-}H_2O$), 315 ($M\text{-}2H_2O$), 307, 279 ($M\text{-}C_2H_4CO_2$), 265, 236, 222, 181, 168, 137, 97, and 79. Ions at *m/z* 333, 315, and 279 are also major fragment ions in the decomposition spectra of authentic PGD_2 and PGE_2 (27). Because of the limited amount of material that was analyzed, it difficult to be certain whether all of the other lower mass ions listed were decomposition ions of *m/z* 351 or background noise. However, analogous to the previously reported decomposition spectra of *m/z* 353, representing the F_2-isoprostane moiety of F_2-isoprostane-containing species of phosphatidylcholine, some of these lower mass ions likely arise from positional isomers of the isoprostane in the mixture (25). These data, therefore, provided further confirmation of the presence of a D_2/E_2-isoprostane esterified to phosphatidylcholine.

Assessment of the Biological Activity of the E_2-isoprostane 8-Epi-PGE_2 in the Kidney—We had shown previously that the F_2-isoprostane, 8-epi-$PGF_{2\alpha}$, exerts potent biological activity in the kidney. Specifically it has been found to be an extremely potent renal vasoconstrictor (5). Recently we have obtained compelling evidence that 8-epi-$PGF_{2\alpha}$ is, in fact, one of the more abundant F_2-isoprostanes that are produced *in vivo* (28). The intermediate endoperoxide that undergoes reduction to yield 8-epi-$PGF_{2\alpha}$ is 8-epi-PGG_2. Since D/E-ring isoprostanes arise from rearrangement of the intermediate endoperoxides, 8-epi-PGG_2 would also be expected to undergo rearrangement to form 8-epi-PGE_2. In this regard, we have been able to obtain preliminary evidence that 8-epi-PGE_2 is, in fact, one of the E-type isoprostanes that is produced *in vivo* by demonstrating that the major *O*-methyloxime isomer of synthetic 8-epi-PGE_2 cochromatographs perfectly on capillary GC with the major peak (★) in the *upper* chromatogram in Fig. 2 (data not shown). The minor *O*-methyloxime isomer chromatograms in the middle of the unresolved series of peaks eluting approximately 25 s earlier. Since we had shown previously that 8-epi-$PGF_{2\alpha}$ is a potent vasoconstrictor of renal vasculature, it was of interest, therefore, to explore whether 8-epi-PGE_2 also exerted biological activity in the kidney.

When infused into the renal artery, 8-epi-PGE_2 decreased both GFR and RPF in a dose-dependent manner (Fig. 8) with no effect on arterial blood pressure. A significant decrease compared with vehicle alone was identified at every dose used in the study and, at 4 μg/kg/min, both parameters decreased by 80%. Simultaneous intravenous administration of SQ29548 at 3 mg/kg/h completely abolished this effect at the intrarenal arterial infusion rate of both 2 and 4 μg of 8-epi-PGE_2/kg/min with no effect on arterial blood pressure.

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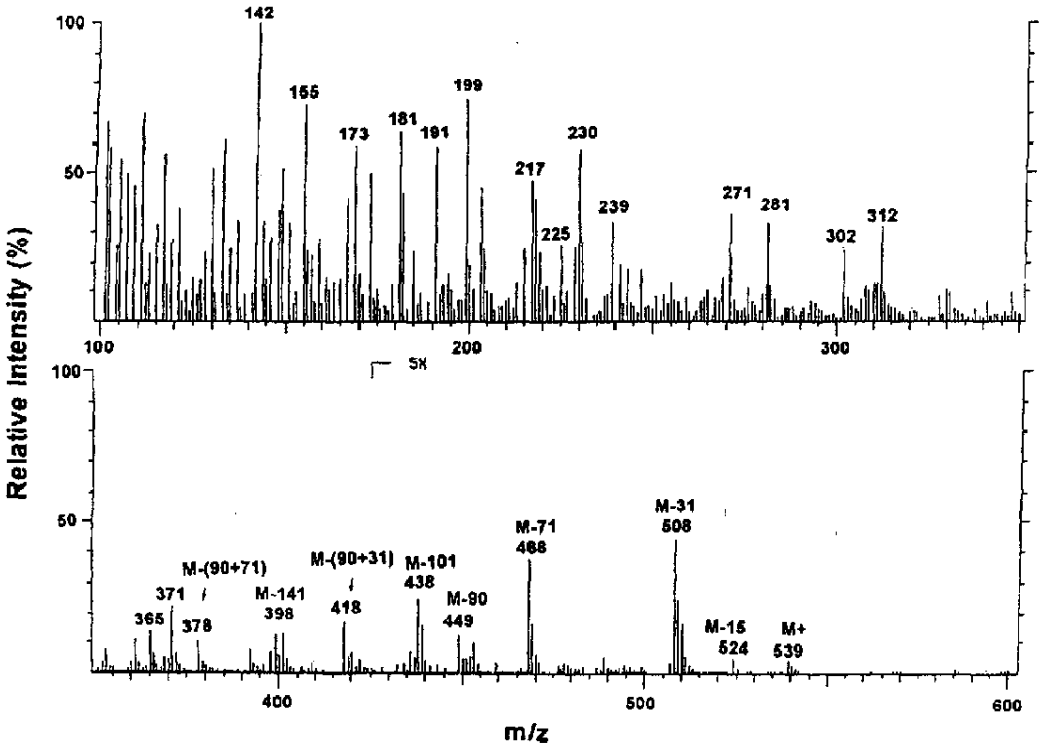

FIG. 6. **A representative electron ionization mass spectrum of D_2/E_2-isoprostanes hydrolyzed from the purified polar lipids that eluted from the HPLC column between 25 and 41 ml as shown in Fig. 5.** Compounds were analyzed as a methyl ester, *O*-methyloxime, TMS ether derivative.

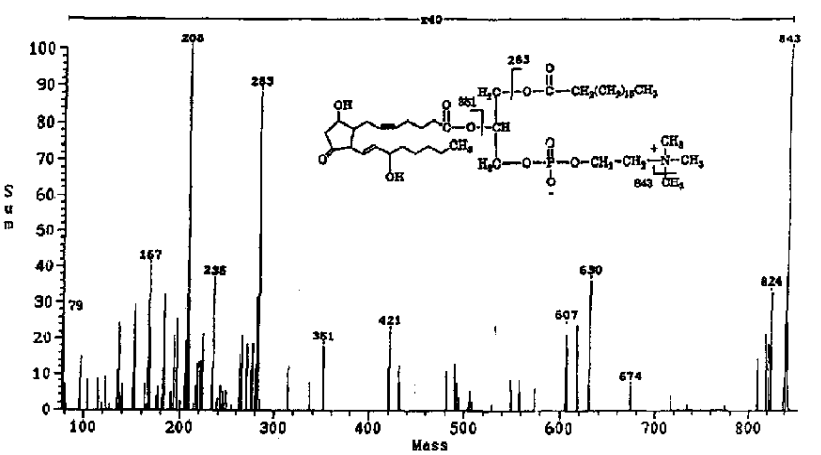

FIG. 7. **Collision-induced dissociation of the M-15 ion, *m/z* 843, in the negative liquid secondary ion mass spectrum of the polar lipids that eluted from the HPLC column between 25 and 41 ml as shown in Fig. 5.**

DISCUSSION

These studies report the discovery that isoprostanes with D/E-type prostane rings are formed *in vivo*. Analogous to the formation of F_2-isoprostanes, D_2/E_2-isoprostanes are also formed *in situ* esterified to phospholipids and released in free form, presumably by a phospholipase(s). The mechanism involved in the formation of D_2/E_2-isoprostanes is outlined in Fig. 9. This mechanism is identical to that outlined previously for the formation of F_2-isoprostanes through the formation of the bicyclic endoperoxide intermediates (11). In the formation of D_2/E_2-isoprostanes, however, the endoperoxides undergo rearrangement to form D/E-ring compounds rather than reduction to form F-ring compounds. Analogous to the formation of F_2-isoprostanes, four regioisomers of D_2/E_2-isoprostanes are formed, each of which can theoretically be comprised of eight racemic diastereomers. It is also conceivable that the endoperoxides might be stabilized in the hydrophobic environment when esterified to phospholipids and may exist for prolonged periods of time before rearranging to D_2/E_2-isoprostanes. However, the finding of high concentrations of D_2/E_2-isoprostanes free in the circulation of rats following administration of CCl_4 establishes that the endoperoxides do undergo rearrangement *in vivo* to form D_2/E_2-isoprostanes.

It has been shown previously that autoxidation of unsatur-

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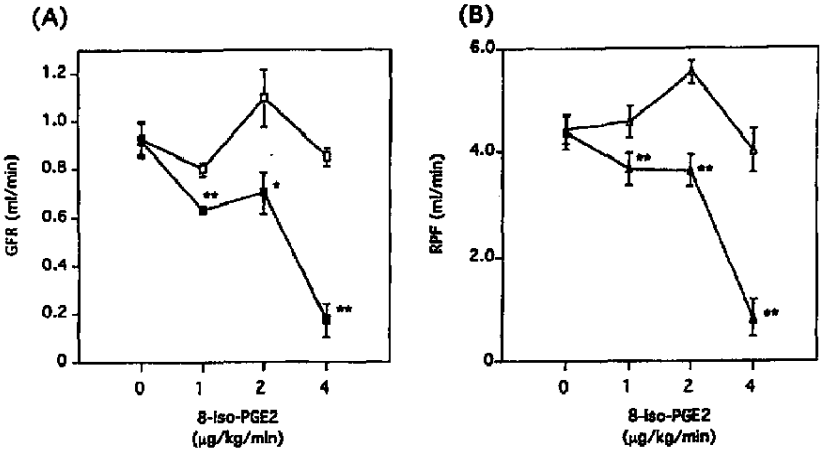

FIG. 8. **Dose-dependent reduction in GFR (*panel A*) and RPF (*panel B*) caused by intrarenal arterial infusion of 8-epi-PGE_2 at rates of 1, 2, and 4 μg/kg/min.** □, GFR in vehicle-infused animal; ■, GFR in 8-epi-PGE_2-infused animal; △, RPF in vehicle-infused animal; ▲, RPF in 8-epi-PGE_2 infused animal. * $p < 0.05$; ** $p < 0.01$ for vehicle infused animal.

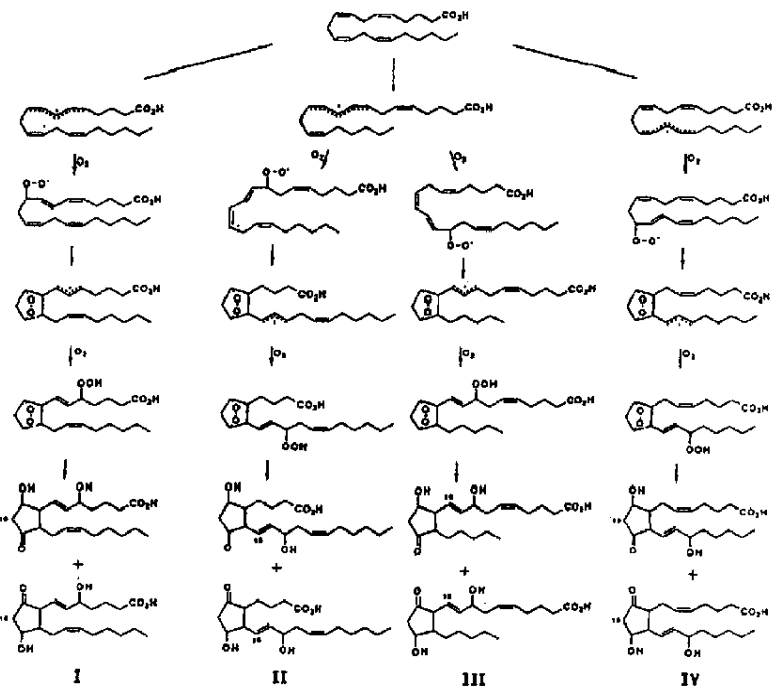

FIG. 9. **Outline of the mechanism involved in the formation of D_2/E_2-isoprostanes.** Four regioisomers of both D_2-isoprostanes and E_2-isoprostanes are produced, each of which can theoretically be comprised of eight racemic diastereomers. For simplicity, stereochemical orientation is not indicated in the figure.

ated fatty acids can result in the formation of compounds with a cyclopentane ring (29). Interestingly, it was found that the side chains of these compounds are almost exclusively oriented *cis* which is in contrast to prostaglandins formed enzymatically by the cyclooxygenase in which the side chains are exclusively oriented *trans* (29). Based on this information, 8-epi prostaglandins would be expected to be formed as isoprostanes. In this regard, recently we obtained evidence that 8-epi-$PGF_{2\alpha}$ is, in fact, one of the F_2-isoprostanes that is produced in abundance *in vivo* (28). Therefore, it would be expected that 8-epi-PGG_2, the endoperoxide intermediate that is reduced to form 8-epi-$PGF_{2\alpha}$, also undergoes rearrangement to form 8-epi-PGE_2. Thus, we examined whether this E_2-isoprostane exhibited biological activity. Since 8-epi-$PGF_{2\alpha}$ is a very potent renal vasoconstrictor (5), we thought it of interest to investigate the effects of 8-epi-PGE_2 on renal hemodynamics. 8-Epi-PGE_2 was also found to be a potent renal vasoconstrictor, albeit somewhat less potent than 8-epi-$PGF_{2\alpha}$ (5). This is an important finding because it suggests that the formation of the D_2/E_2-isoprostanes is not simply a phenomenon of biochemical curiosity, but one that may also have biological relevance. The finding that 8-epi-PGE_2 is a vasoconstrictor of renal vasculature is somewhat unexpected since PGE_2 derived via the cyclooxygenase is a vasodilator (30). In contrast, both $PGF_{2\alpha}$ derived via the cyclooxygenase and the F_2-isoprostane 8-epi-$PGF_{2\alpha}$ are vasoconstrictors (5). Thus, one might anticipate that 8-epi-PGE_2 and 8-epi-$PGF_{2\alpha}$ would also have opposing biological effects on vascular smooth muscle. However, this may be an oversimplistic expectation based on the structure/function relationships of prostaglandin molecules. We reported previously that the renal vasoconstricting actions of 8-epi-$PGF_{2\alpha}$ could be abrogated by SQ29548, a thromboxane receptor antagonist, suggesting that the vascular actions of 8-epi-$PGF_{2\alpha}$ are mediated via an interaction with thromboxane receptors (16). This was an interest-

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ing finding in that thromboxane receptors have not been implicated in the vasoconstricting activity of $PGF_{2\alpha}$. More recently, however, we reported that 8-epi-$PGF_{2\alpha}$ appears to interact with a distinct novel receptor on vascular smooth muscle, which is apparently antagonized by SQ29548, which may be similar, but different, from the thromboxane receptor (31). Thus, simple inversion of the stereochemistry of the upper side chain of $PGF_{2\alpha}$ may be a key determinant of receptor interaction. Of interest, we found that the vasoconstricting actions of 8-epi-PGE_2 in the kidney could also be abrogated by SQ29548. Future studies investigating whether 8-epi-PGE_2 also interacts with the same distinct receptor as 8-epi-$PGF_{2\alpha}$ (owing to the fact that the stereochemistry of the upper side chain is inverted), or interacts with the thromboxane receptor, will be of considerable interest. In this regard, studies investigating further the spectrum of the biological activity of 8-epi-PGE_2 and the biological actions of other D_2/E_2-isoprostanes will also be of interest and of potential importance since these compounds may participate as mediators in the pathophysiology of oxidant injury.

It should be noted that the quantities of D_2/E_2-isoprostanes that are formed *in vivo* are only slightly less than the amounts of F_2-isoprostanes formed. Since the levels of F_2-isoprostanes in normal human biological fluids are approximately an order of magnitude higher than cyclooxygenase derived prostaglandins (5), the amounts of D_2/E_2-isoprostanes which are produced *in vivo* are not trivial. At present, enzymes and/or nonenzymatic substances that may be involved in the reduction of the isoprostane endoperoxide intermediates *in vivo* to F-ring isoprostanes remain to be identified. Nonetheless, understanding factors regulating the production of D/E-ring isoprostanes in relation to F-ring isoprostanes might be important since changes in the relative abundance of these different isoprostanes may have different biological consequences owing to potential variations in their biological actions.

It should also be mentioned that there are potentially important biological ramifications associated with the formation of D_2/E_2-isoprostanes esterified to phospholipids. We reported previously that molecular modeling of phospholipids with F_2-isoprostanes esterified at the *sn*-2 position revealed them to be extremely distorted molecules (7). Thus, the formation of isoprostane-containing phospholipids in settings of oxidant stress may have deleterious effects on membrane fluidity and integrity, well recognized sequelae of oxidant injury. Since we have now discovered that D_2/E_2-isoprostanes are also formed esterified to phospholipids in only slightly less abundance compared with F_2-isoprostanes, the total quantities of isoprostane-containing phospholipids that may be formed in settings of free radical injury are substantially greater than thought previously.

We demonstrated that D_2/E_2-isoprostane-containing phospholipids are substrates for bee venom phospholipase *in vitro*. We also were able to detect free D_2/E_2-isoprostanes in the circulation of rats treated with CCl_4 to induce lipid peroxidation. Presumably the free compounds arose primarily from hydrolysis of isoprostane-containing phospholipids. The type(s) of mammalian phospholipase responsible for hydrolysis of D_2/E_2-isoprostanes from phospholipids has not yet been identified. However, understanding how this process is regulated is of importance. As discussed above, isoprostane-containing phospholipids are very distorted molecules that should have profound effects on the integrity and fluidity of cellular membranes. However, we have demonstrated that once released in free form, D_2/E_2-isoprostanes are capable of exerting biological activity. How these two processes are balanced, therefore, will determine the overall net biological ramifications associated with the formation of isoprostanes *in vivo*.

In summary, we report the discovery that D_2/E_2-isoprostanes capable of exerting potent biological activity are formed *in vivo* as products of nonenzymatic free radical-catalyzed lipid peroxidation. Analogous to the formation of F_2-isoprostanes, D_2/E_2-isoprostanes are formed *in situ* esterified to phospholipids and subsequently released in free form. Further understanding the biological consequences of the formation of these novel compounds and factors that can influence their formation may provide valuable insights into the pathophysiology of oxidant injury.

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